CONTENTS

DIAGNOSIS OF HONEY BEE DISEASES, PARASITES, AND PESTS

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INTRODUCTION

Bee disease inspection is an important part of beekeeping. Apiary inspectors and beekeepers must be able to recognize bee diseases and parasites and to differentiate the major diseases from the less important ones. The purpose of this publication is to acquaint readers with some of the techniques used for diagnosing bee diseases. We realize that different laboratory methods are used by others, but this publication contains only those used in the Bio-environmental Bee Laboratory.

DISEASES OF THE LARVAE

Diagnosing Techniques

The correct diagnosis of any bee disease depends on the sample. In the case of brood disease, examine the whole comb before selecting a specimen. Table 1 shows some of the more important symptoms in diagnosing a brood disease under field conditions. Always look first for larvae or pupae with typical symptoms of American foulbrood disease.

Do not crush any larva or pupa before carefully examining the specimen. Note whether the sample is a larva or pupa, its position in the cell, and its color. Remember that no one symptom should be taken as absolute evidence in the diagnosis of any disease. Since laboratory verification of sacbrood and paralysis disease requires either special antisera or an electron microscope, most laboratories rely on gross symptoms for the diagnosis.

Many times the larval remains are difficult to locate because the combs may be crushed in transit. Scale material can be simply and easily located by using long-wave ultraviolet or near-ultraviolet light. Exposure between 3100 and 4000 angstrom units will fluoresce scale material. Use some discretion with this technique because honey and pollen will also fluoresce.

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Most bee diseases can be diagnosed, with a little practice, by observing the pathogens with a light microscope. However, in some cases, correct diagnosis requires cultural techniques in which the pathogen is grown on artifical media and identified by biochemical tests. (See appendix, "Laboratory Cultivation of Micro-organisms Associated With Honey Bees.")

Modified Hanging Drop

Preparing the Smear—Smears of suspected larval material are mixed with a drop of distilled water on a clean cover slip until a slightly cloudy film is obtained. Matchsticks or smears on paper can first be resuspended in water and then smeared on the cover slip. The cover slip with the smear side up is then heat-fixed under a heat lamp or over an open flame. Smear enough oil on a clean microscope slide equivalent to an area about twice the width of the cover slip. You can do this while your smear is being heat-fixed. Be certain that the smear is completely dry before proceeding.

Staining the Smear—Stain the smear with carbol fuchsin (see appendix) for 5 to 7 seconds. Enough stain should be placed on the cover slip to cover the entire smear. Be careful to stain the smear and not the clear side. The excess stain is then washed off with water.

Preparing the Slide—While the cover slip is still wet, quickly place it, smear side down, on the pre-

Table 1.—Comparison of symptoms of various brood diseases of honey bees

Symptom	American foulbrood	European foulbrood	Sacbrood	Chalk brood
Appearance of brood comb	Sealed brood. Discolored, sunken, or punctured cappings.	Unsealed brood. Some sealed brood in advanced cases with discolored, sunken, or punctured cappings.	Sealed brood. Scattered cells with punctured cappings, often with 2 holes.	Sealed and unsealed brood. Affected larvae usually on outer fringes.
Age of dead brood	Usually older sealed larvae or young pupae. Upright in cells.	Usually young unsealed larvae; occasionally older sealed larvae. Typically in coiled stage.	Usually older sealed larvae; occasionally young unsealed larvae. Upright in cells.	Usually older larvae. Upright in cells.
Color of dead brood	Dull white, becoming light brown, coffee brown to dark brown, or almost black.	Dull white, becoming yellowish white to brown, dark brown, or almost black.	Grayish or straw-colored, becoming brown, grayish black, or black; head end darker.	Chalk white. Sometimes mottled with black spots.
Consistency of dead brood	Soft, becoming sticky to ropy.	Watery; rarely sticky or ropy. Granular.	Watery and granular; tough skin forms a sac.	Watery.
Odor of dead brood	Slight to pronounced glue odor to gluepot odor.	Slightly to penetratingly sour.	None to slightly sour.	Slight, nonobjectionable.
Scale characteristics	Uniformly lies flat on lower side of cell. Adheres tightly to cell wall. Fine, threadlike tongue of dead pupae may be present. Head lies flat. Black in color.	Usually twisted in cell. Does not adhere tightly to cell wall. Rubbery. Black in color.	Head prominently curled toward center of cell. Does not adhere tightly to cell wall. Rough texture. Brittle. Black in color.	Does not adhere to cell wall. Brittle. Chalky white in color.

viously prepared slide smeared with the immersion oil. Then gently blot the cover slip and slide between some paper towels or blotters. The slide is now ready for examination under the microscope.

Examining the Slide—An oil immersion objective is necessary to examine the smear. If you are unsure about using the oil immersion objective, read "The Microscope" in the appendix before proceeding.

The modified hanging drop technique is especially useful for differentiating between American and European foulbrood disease. Look for the areas where the water has been trapped between pockets of oil. Often it is necessary to refocus the microscope to detect the presence of floating spores. Only spores of *Bacillus larvae* show a Brownian movement. This is an extremely valuable diagnostic feature, since spores of *B. alvei* in a similar mount generally remain attached to the cover slip. This, taken together with the characteristic morphology of the spores, usually allows an almost immediate diagnosis. The technique also lends itself to direct examination for *Streptococcus pluton* if *B. alvei* is not present.

Standard Smear

Preparing the Smear—Proceed as in hanging drop method except make the smear directly on microscope slide. Heat-fix smear over open flame or under heat lamp. Be certain that the smear is completely dry before proceeding.

Staining the Smear—Stain the smear as described in previous section or with any other simple bacterial stain.

Preparing the Slide—Air-dry the stained smear.

Examining the Slide—An oil immersion objective is necessary to properly examine the smear. No cover slip is necessary. Place a drop of immersion oil directly on the smear.

Foulbrood Diseases

See table 2 for biochemical tests to differentiate *Bacillus* spp. and also see figures 1 and 2.

Bacillus larvae—The bacterium that causes American foulbrood is a slender rod with ends slightly rounded and a tendency to grow in chains. It varies greatly in length, from about 2.5 to 5 micrometers

Table 2.–Differentiation of *Bacillus* spp. encountered in honey bees

Species	Catalase	Voges-Proskauer	Starch hydrolysis	Nitrate reduction	Growth in nutrient broth
B. larvae	-	-	-	+	-
B. alvei	+	+	+	-	+
B. laterosporus	+	-	-	+	+
B. pulvifaciens	-	-	-	+	+

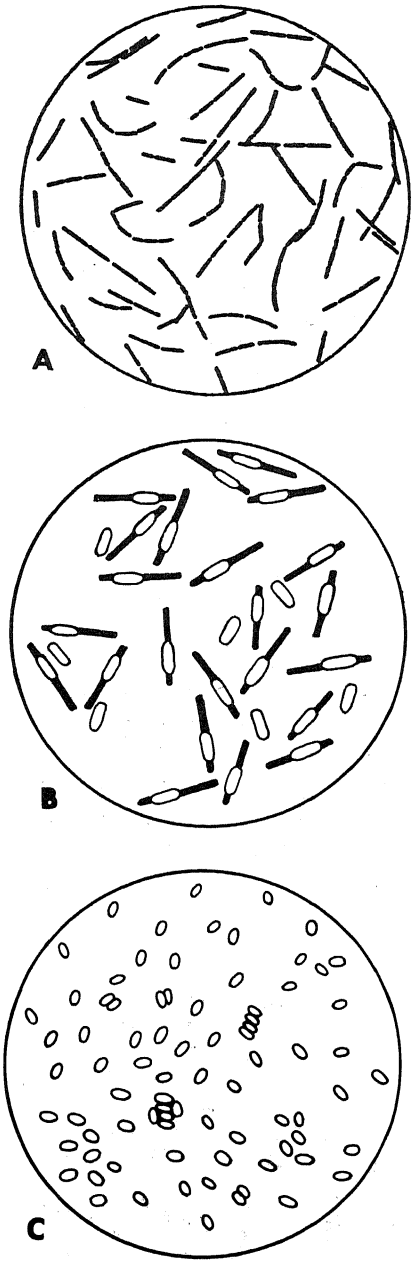


Figure 1.—Sketches of bacterium which causes American foulbrood disease (not to scale): (A) *Bacillus larvae* vegetative cells, (B) *Bacillus larvae* spore formation, (C) *Bacillus larvae* spores.

(μm) and is about 0.5 μm in width. The spore form is an oval-shaped organism approximately twice as long as it is wide, measuring about 0.6 by 1.3 μm. It will be observed as a reddish-purple stained spore somewhat deeper in color about the cell wall and quite clear in the center.

Streptococcus pluton—This bacterium is the cause of European foulbrood. It is a short, nonspore-forming, lancet-shaped cell occurring singly, in pairs, or in chains. The bacterium measures 0.5 to 0.7 by 1.0 μm (see fig. 2).

Bacillus alvei—Although this organism is not the cause of any bee disease, its presence can be used as an indicator for diagnosing European foulbrood. *B. alvei* is a rod-shaped organism measuring approximately 0.5 to 0.8 μm in width and 2.0 to 5.0 μm in length. Spores are produced and measure 0.8 by 1.8 to 2.2 μm .

Bacillus laterosporus—Formerly this organism was named *Bacillus orpheus.* Occasionally, it is found in larvae with European foulbrood disease. Rods measure 0.5 to 0.8 μm by 2.0 to 5.0 μm and the spores 1.0 to 1.3 μm by 1.2 to 1.5 μm . It will be observed as an elongated oval-shaped organism, rounded at both ends, stained very heavily along one side and the two ends. The clear portion and the finely outlined cell wall are the spore. Some of the elongated rod forms are present and are characterized chiefly by the absence of the spore or clear portion in the cell.

Achromobacter (Bacterium) eurydice—This is another bacterium that is frequently found in cases of European foulbrood disease. It is a small, nonspore-forming, slender rod with slightly rounded ends occurring in pairs or singly. The cells of *A. eurydice* are 0.5 to 1.4 μm in length by 0.4 to 0.7 μm in width.

Sacbrood Disease

Since there are other diseases that on gross appearance could be confused with American and European foulbrood, microscopic examination is important. Sacbrood-diseased larvae are relatively free from bacteria. However, do not use the absence

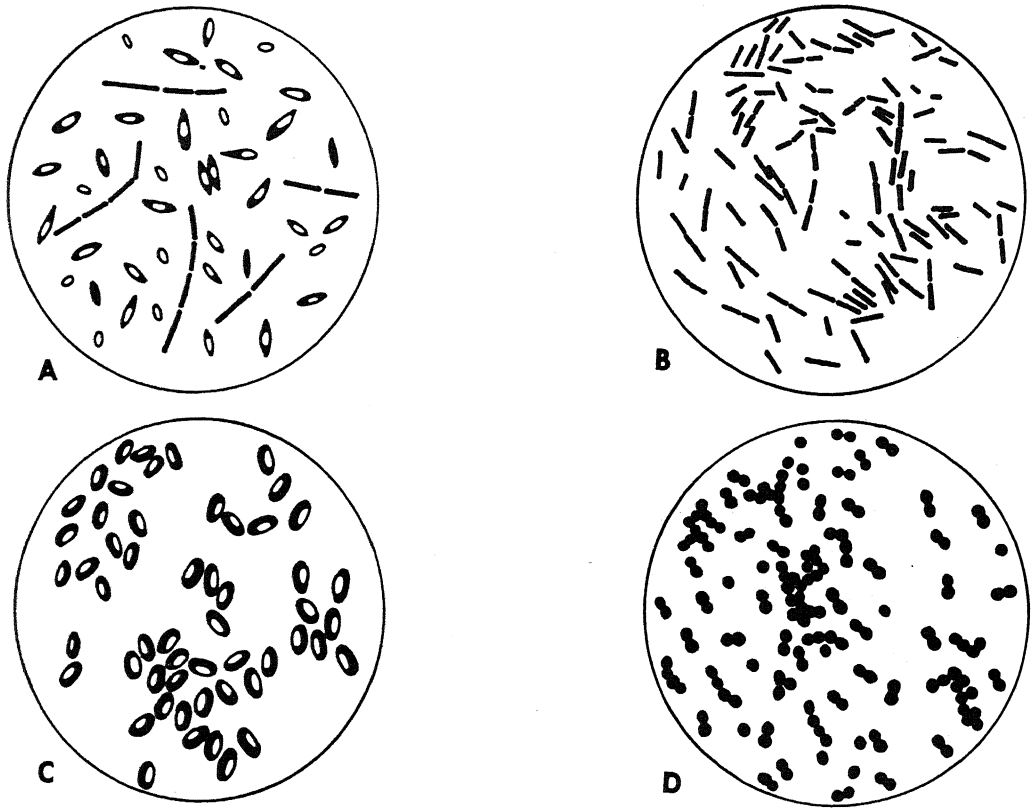


Figure 2.—Sketches of bacteria which cause or are associated with diseases of the honey bee (not to scale): (A) *Bacillus alvei* spore formation, (B) *Achromobacter eurydice*, (C) *Bacillus laterosporus*, (D) *Streptococcus pluton*.

of bacteria as the sole symptom in the diagnosis of sacbrood disease. The causative virus, *Morator aetatulas*, cannot be seen even with an oil immersion objective of a light microscope.

Powdery Scale Disease

In rare cases you may also encounter powdery scale disease. This is strictly a larval disease. The resulting scale is powdery and this is useful in diagnosis. The pathogenic agent of powdery scale disease is a spore-forming rod, *Bacillus pulvifaciens*. This bacterium does not show Brownian movements in the modified hanging drop method. The vegetative rods measure 0.3 to 0.6 μm by 1.5 to 3.0 μm (see fig. 3). The spores measure 1.0 by 1.3 to 1.5 μm . (See also appendix, "Laboratory Cultivation of Microorganisms Associated With Honey Bees.")

Chalk Brood Disease

For any disease that you suspect to be of fungal origin, prepare a wet mount. Generally, all that is required is to suspend a portion of the sample in water and macerate. Place a loopful of this suspension on a microscope slide and carefully drop the cover slip on the suspension to avoid air bubbles. No stain is required and the slide should be examined with only the dry objectives (no higher than 43X).

In this disease, gross symptoms are important because the untrained person will find it difficult to verify the identity of *Ascosphaera apis*, the cause of

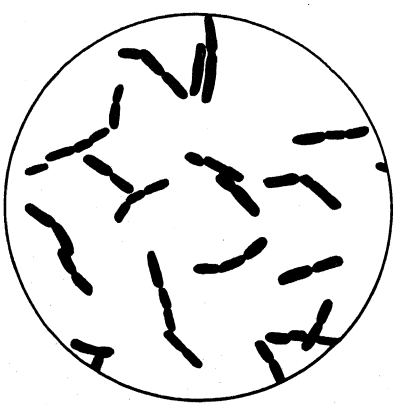


Figure 3.—Vegetative rods of *Bacillus pulvifaciens*.

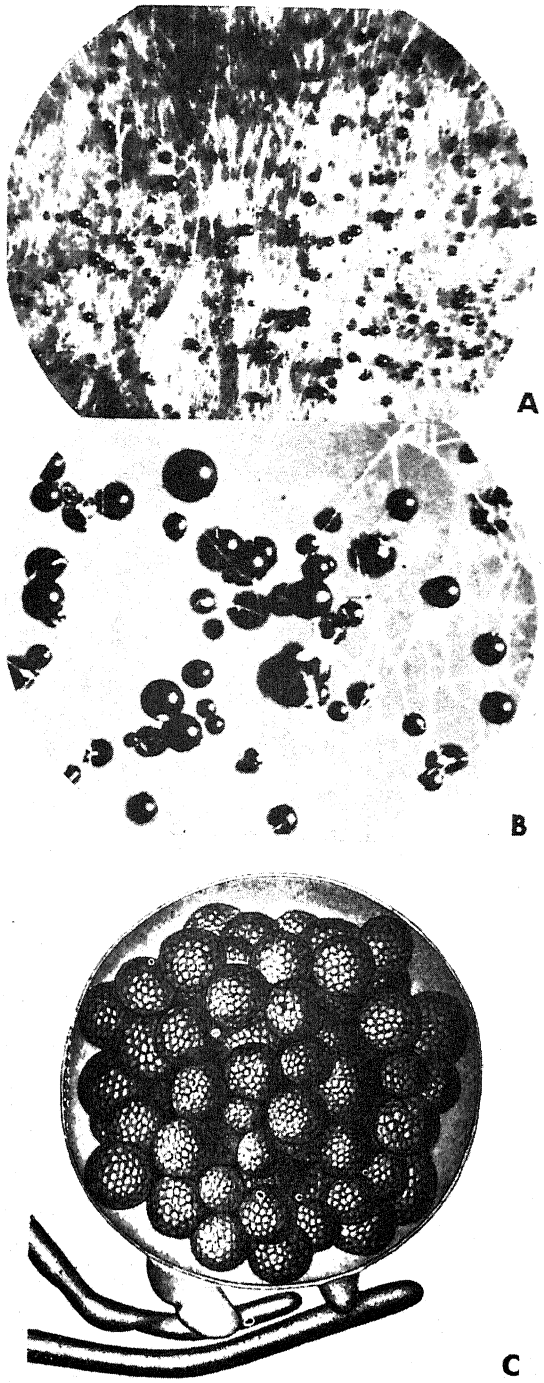


Figure 4.—Photographs of a growing culture of *Ascosphaera apis*: (A) 20X, and (B) 80X; (C) drawing of a sporocyst of *A. apis* containing many spores.

chalk brood disease. This fungus has both filamentous and spore stages (see fig. 4). The most useful stage for identification is the spore cyst, measuring 47-140 μm in diameter. Spore balls enclosed within the cyst range from 9-19 μm in diameter, while the individual spores are 3.0 to 4.0 μm by 1.4 to 2.0μm in size. (See also appendix, "Laboratory Cultivation of Micro-organisms Associated With Honey Bees.")

Stonebrood Disease

This bee disease can be caused by more than one fungus; the primary cause is *Aspergillus flavus* (fig. 5).

Positive laboratory diagnosis of this disease requires some skill. Fungi, in general, are difficult to identify unless you have some experience in this speciality. For this reason, it is important to look for gross symptoms.

A. flavus can be pathogenic to larvae and adult bees. In both cases, the diseases caused by this fungus are considered quite rare. In adults, the first symptom is the weak and restless appearance of the bees. Eventually the bees are unable to fly or right themselves.

Stonebrood in larvae is quite difficult to identify in the early stages of infection. After death, the abdomen of the affected bee becomes hardened and quite difficult to crush, hence the name stonebrood. A wet mount prepared from the larva shows mycelium penetrating throughout the entire insect. Eventually, the fungus erupts from the integument of the insect and forms a false skin (mummy). At this stage, the larva may be covered with a green powdery substance (fungus spores). Usually, the spores are formed first, most abundantly, near the head end of the dead larva.

Since many fungi inhabit hives of honey bees, it is important to correctly identify *A. flavus*. Other species of *Aspergilli* also cause stonebrood disease.

Nosema Disease

Nosema disease is caused by the protozoan, *Nosema apis* (Zander). Nosema spores are large oval bodies about 4-6 μm in length by 2-4 μm in width (fig. 6). They possess a thin polar filament which is rarely seen extruding from the spore body. This filament, when extruded, is several times longer than the spore. A microscope is required to detect spores of *N. apis*. No single symptom typifies this disease. Affected bees may be unable to fly; consequently, these bees may crawl about at the entrance to the colony. The best time to find spores in the bees is at the beginning of the flying season after winter confinement.

There is no reliable field diagnostic symptom. However, in severe cases it is possible to see differences between healthy and Nosema-diseased bees. In healthy bees, the individual circular constrictions on the ventriculus are clearly seen, and in diseased bees the constrictions are not clear (fig. 7).

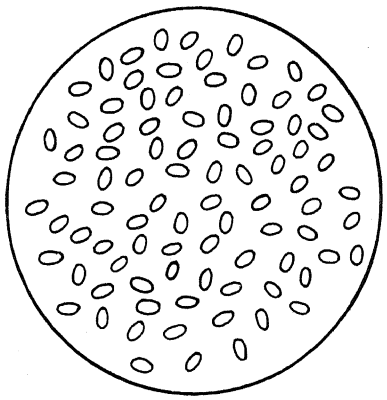


Figure 6.—Nosema spores as they appear in a wet mount.

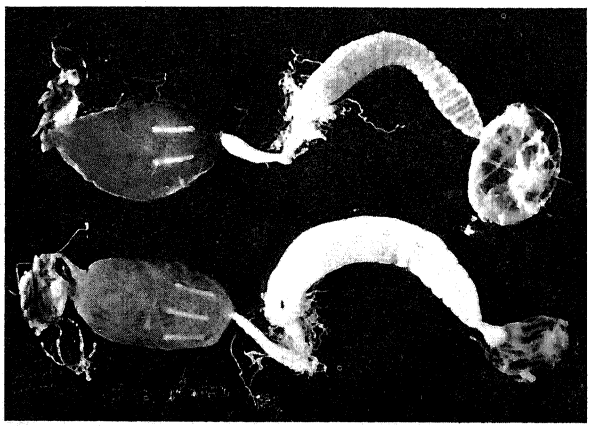


Figure 7.—Digestive tract from (top) a healthy honey bee and (bottom) a bee with Nosema disease.

Methods for Examining—The sample and the sampling technique are as important as the spore-counting technique. If possible, a sample of 25 bees should be used; however, fewer bees may be used, though no less than 10. Also since newly emerged adults do not show infection, only older workers should make up the sample. These bees should be taken from the hive entrance just before or after flight. If this is not possible because of inclement weather, sampling should be done under the top board or in the cluster.

After the bees are immobilized by freezing or CO^2, they are placed in a concave dish along with 1 milliliter (ml) of water per bee and ground with the rounded end of a clean test tube. (These tubes and dishes should be washed thoroughly before reusing.) Preliminary examination of the suspension can be made using the wet mount technique. This can save you considerable time; in many cases, examination to determine the presence or absence of Nosema disease is all that is necessary. If *N. apis* spores are found, you can then use one of the two following counting procedures.

Preparing a Wet Mount—Place a drop of the suspension containing the macerated bee abdomens on a microscope slide. Then carefully place the cover slip on the suspension. The proper method for placement of the cover slip is to touch one of its edges to the slide at a 45° angle so that the drop of water touches it and the slide. The cover slip is then released; as it falls, air is forced from between it and the slide. If this is done properly, no air bubbles will form. The wet mount should be examined with either the low or high dry objective of the microscope.

Counting Nosema Spores—Two methods of counting Nosema spores that are in general usage are presented here. The first is a modification of the method used in the laboratories to count human blood cells and incorporates a special piece of equipment called a hemocytometer (fig. 8). It consists of a special cover glass and a chamber, ruled for ease in counting, that holds a specific volume of fluid. These chambers cost about $15 and are available from scientific supply houses. Before use, the chamber should be cleaned and freed from lint. To clean the chamber, dip it in soapy water, rinse in clean water, flood it with alcohol, and finally dry the chamber with a lint-free cloth.

A sample of the spore suspension is removed, preferably with a loop, and placed under the cover glass. When the droplet touches the chamber and cover glass, it will flow under the cover glass, thereby

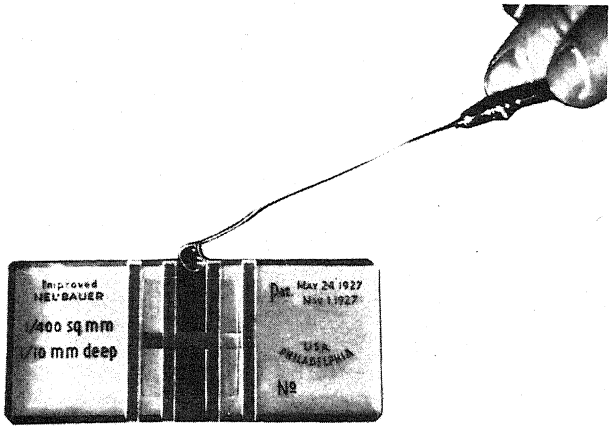


Figure 8.—Loading a hemocytometer.

filling the chamber and insuring the correct volume. Do not overload or allow fluid to run down into the moats on either side. Also, make sure no air bubbles are under the cover glass. Allow about 3 minutes for the spores to settle before counting. But first, find the ruled area and focus the microscope on the spores so they are sharply defined. These squares are arranged in groups of 16 with each group bounded by double lines (fig. 9).

Count all the spores in the block bounded by the double lines shown in figure 9. Include in the counts all spores that are in the area bounded by lines A and B. The spores on the two A lines should be counted, but do not count the spores that touch the B lines. The pattern of counting and of moving to the next area of the chamber should become routine to insure against skips or against counting the same area twice. To obtain a good average, count five blocks each containing 16 of the small squares. See figure 10 to help you in selecting the five blocks.

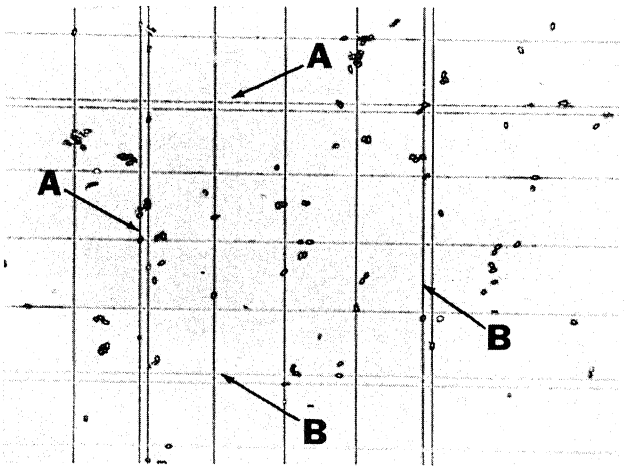


Figure 9.—Sixteen squares enclosed by the double lines.

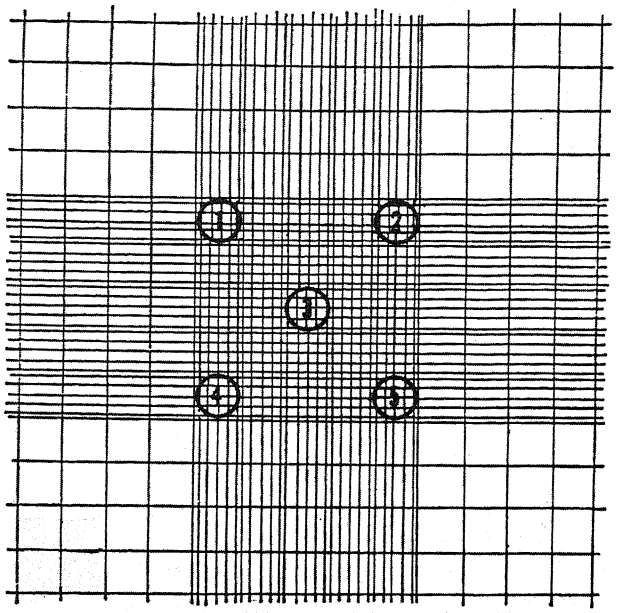


Figure 10.—The selection of the five blocks of 16 squares bounded by the double lines.

Since each of the small squares in the chamber has dimensions of 0.05 by 0.05 by 0.1 mm, the total volume is 0.00025 mm^3, that is, 1/4000 of 1 mm^3. Therefore, find the average number of spores per square and multiply this figure by 4,000 to obtain the number of spores per cubic millimeter. Then to determine the number per cubic centimeter (cm^3) or milliliter, multiply by 1,000 the number per cubic millimeter. If you started with 1 ml of water for each ground-up bee, the number of spores per cubic centimeter is equal to the number per individual bee. Use the following equation to determine the number of spores per bee if you started with the equivalent of 1 ml of water per bee.

$$\frac{\text{Total No. spores counted}}{80} \times \frac{4 \times 10^6}{1} = \text{No. spores/bee}$$

In certain instances, the spores may be so numerous that accurate counts cannot be made. Then the suspension must be diluted. Usually, a dilution of 1 part spore suspension to either 1 or 9 parts water (which gives a dilution factor of 2 or 10, respectively) will be sufficient to allow accurate counts. This factor must then be incorporated into the equation as in the following example for a dilution factor of 10.

$$\frac{\text{Total No. spores counted}}{\text{No. squares counted}} \times \frac{10}{1} \times \frac{4 \times 10^6}{1} = \text{No. spores/ml}$$

Some precautions and possible sources of error that should be avoided:

1. Shake the spore suspension before removing the loop subsample to insure even distribution.
2. Flame the loop before using it to load the chamber.
3. Use a clean counting chamber.
4. Do not make a count if air bubbles are present or if there is an obviously unequal distribution of spores in the chamber.
5. Allow the spores to settle in the counting chamber for 3 minutes.
6. Count before the sample begins to dry in the chamber.

The second method of estimating the number of spores per volume (or per bee) can be done without the hemocytometer. However, the method is similar since it involves counting the spores in a small volume and relating the number to a larger volume.

Make a wet mount from the spore suspension by placing a loopful of the fluid on a clean glass slide. (The loop must deliver a known volume of fluid; calibrated loops may be made or purchased. We recommend one that delivers 0.01 ml.) A clean cover slip of known area is then placed over the sample in such a way as to avoid air bubbles. Examine the preparation to see whether the spores are evenly distributed; then select the microscopic objective (the high dry objective is recommended). Record the numbers of spores from five different fields of view selected at random and obtain the average per field. Remember that the area of the field changes with each different objective. The area of the fields can be determined with a stage micrometer.

If the calibrated loop delivers 0.01 ml, the following equation may be used to determine the number of spores per milliliter.

$$\frac{\text{Average No. spores/field}}{1} \times \frac{\text{Area of cover slip}}{\text{Area of field}} \times \frac{100}{1} = \text{No. spores/ml}$$

All of the precautions for use of the hemocytometer also apply to this technique.

Coprological Test for Queens—Nosema disease in a queen is especially critical to honey bee colonies because the disease can impair her egg-laying capacity and result in her supersedure. Furthermore, ...n can be a major source of Nosema disease ...y.

...tion for Nosema disease is relatively ...e sacrificed. However, where ...lved the task becomes more ...you may want to export that ...se her in some test; con- ...eep her alive.

Coprological examination of the queen is the only direct method. Examination of attendant bees is used most commonly, but it is indirect and not a reliable test. There are no precise data on the reliability of coprological examinations but it should exceed 85 percent.

Suspect queens are brought into the laboratory and held in small petri dishes or in glass tubes. The queens are allowed to walk freely and usually defecate within 1 hour. Queens should be returned to hives immediately to avoid the possibility of not being accepted. Remember, queen feces are colorless so they will appear as a drop of clear liquid. Transfer this clear liquid with a pipet or capillary tube to a microscope slide and place a cover glass over the drop. The slide is now ready for examination with the high dry objective.

Paralysis Disease

Paralysis of adult honey bees is a condition that can be caused by a communicable agent or toxic substance. In this context, we will refer to paralysis as a disease of adult bees caused by a virus. This disease occurs through the entire bee season and it seldom destroys a colony but, in severe cases, can reduce honey production.

Bees affected by this disease are usually found on the top bars of the combs, appear to tremble uncontrollably, and are unable to fly. When this condition is serious, large numbers of bees can be found crawling out of the colony entrance. Affected bees are often hairless and appear to have no control of their wings and legs (fig. 11). Abdomens of affected bees may be dark, shiny, or greasy.

Ideally, the diagnosis of paralysis disease is made using serological techniques. Since this is beyond the capability of most laboratories, diagnosis of the disease is made by observing the individual bees and also the colony behavior where possible.

Most laboratories can diagnose paralysis disease by reproducing the disease symptoms in caged bees. Generally, bees can be infected by spraying, feeding, or injecting an extract made from diseased bees. The extract is prepared by macerating the equivalent of one bee in 1 ml of water. The extract is then centrifuged to eliminate the large suspended matter and then filter sterilized.

For spraying, use approximately 2.5 ml of the extract per bee. In feeding trials, 2 ml of the extract should be mixed with an equal volume of sugar

Figure 11.—Healthy bee (above) and paralysis-diseased bee (below). Note the lack of hair on the thorax of the diseased bee and also its dark, shiny appearance, unhooked wings, and distended mouthparts.

syrup and fed to 20 caged bees. Injection is probably the most tedious means of transmitting the disease. Each bee is inoculated with 1 microliter of the suspension using a micrometer syringe fitted with a 30-gauge hypodermic needle. The bees should first be anesthetized with carbon dioxide and then inoculated. Only the tip of the needle should be inserted into the hemocoele through a dorsal abdominal intersegmental membrane.

Bees should be held in cages at 30° C and fed syrup as needed. The symptoms of paralysis disease should be visible after 6 days. Appropriate controls should be treated with extracts of healthy bees.

Septicemia Disease

This disease is caused by the bacterium, *Pseudomonas apiseptica.* Only adult bees are affected by this disease. The bees that die from septicemia often have a putrid odor. The muscles of the thorax decay rapidly and the body, legs, wings, and antennae fall apart when handled. Laboratory diagnosis can be accomplished by preparing a water extract and inoculating healthy bees. Bees with septicemia die rapidly, within a day or two, and exhibit the typical odor and break-apart symptoms. Extracts of the dead bees can also be used to isolate pure cultures of the organism for identification. (See fig. 12.) For culture medium, see "Laboratory Cultivation of Microorganisms Associated With Honey Bees."

Amoeba Disease

The parasitic amoeba, *Malpighamoeba mellificae,* occurs only in the adult bee. No gross symptoms are explicit for amoeba disease. This disease has also been found in bees infected with *Nosema apis.* Amoeba disease has a disease cycle similar to that of Nosema.

Diagnosis of this disease requires careful removal of the Malpighian tubules, which are long, threadlike projections originating at the junction of the midgut and the hindgut. The tubules can be placed directly in a drop of water on a microscope slide. Then place a thin cover slip (No. 1 thickness) directly over the tubules. Gently apply uniform pressure to the cover slip to obtain a flat surface for microscopic examination. The amoeba cysts examined with oil immersion objectives will appear as tiny droplets of oil in water. The diseased tubules will be packed full of these amoeba cysts (fig. 13).

Gregarine Disease

There are four nominal gregarines that are associated with adult honey bees. They are: *Monoica apis, Apigregarina stammeri, Acuta rousseaui, and Leidyana apis.* No gross symptoms have been described for the gregarines. Diagnosis of this condition can only be made with a microscope (fig. 14). The extent of infections in honey bees in the United States is unknown.

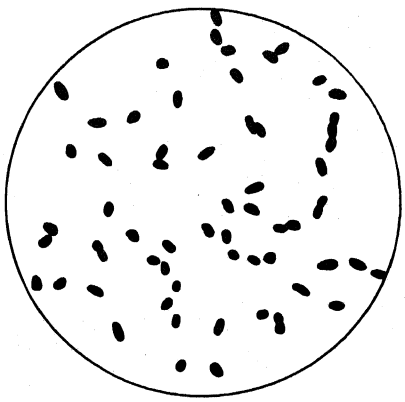


Figure 12.—*Pseudomonas apiseptica.*

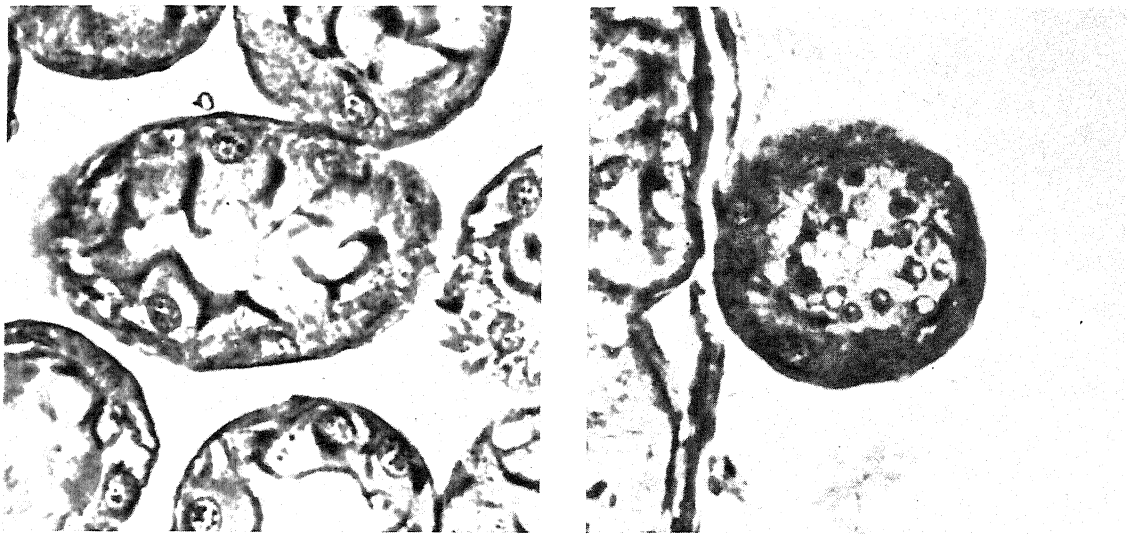


Figure 13.—Cross-section of Malpighian tubules. On the left is a healthy tubule, and on the right, a tubule containing cysts of *Malpighamoeba mellificae.*

Bee "Louse"

The bee "louse" is not really a louse, but a wingless dipteran, *Braula coeca.* The insects are about 1.5 mm in length and 1 mm in width. Adults have vestigial compound eyes, short sunken antennae, and neither wings nor halteres (fig. 15). Larvae of *B. coeca* are 0.5 mm to 1.5 mm in length and eggs measure 1 mm by 0.5 mm.

Adult *B. coeca* attach themselves to adult bees and do not seem to cause them any harm. Probably the only damage caused by these insects is while they are

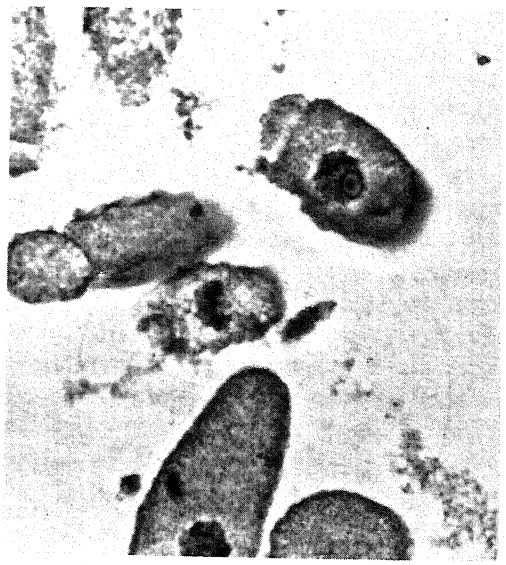
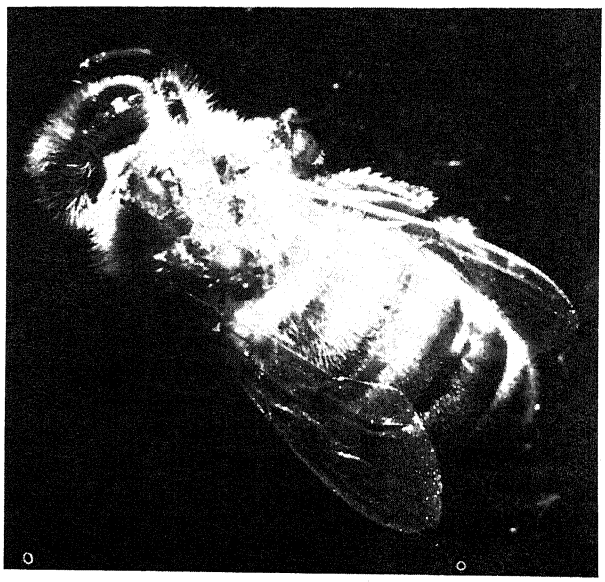


Figure 14.—Gregarine.



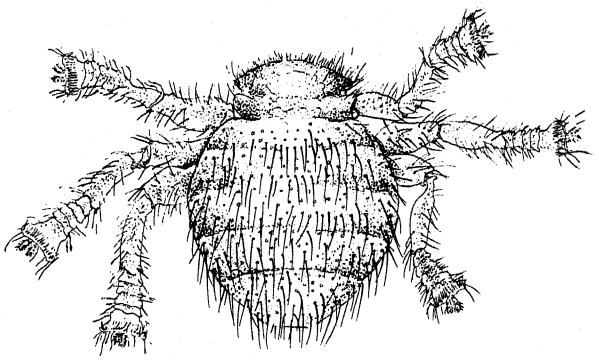


Figure 15.—*Braula coeca*: Above, on thorax of worker bee; below, close-up drawing.

larvae. The larvae tunnel through the cappings and detract from the appearance of comb honey.

Mites

Honey bees are affected by both external and internal mites. It is important to note that not all external mites are harmful to honey bees. *Acarapis woodi* (Rennie) is the only internal mite found in the honey bee. Although no parasitic mites have been found on honey bees in the United States, it is well to be familiar with the techniques for diagnosing mite disease.

Acarine Disease—Acarine disease, sometimes referred to as acariose, is caused by the parasitic mite, *A. woodi*. The female mite enters the body of the honey bee through the large first thoracic spiracles; queens, workers and drones are equally susceptible to attack. Entrance into the young bee must be made within 5 days of its emergence or it will not be attacked by the mite. After the female mite lays her eggs in the trachea, they hatch and the young mites presumably feed on the hemolymph of the bee by piercing the tracheal wall with their mouthparts. These punctures cause the injured tissues to become melanized, which results in a dark brown spotting of the tracheal wall, a characteristic of heavy mite infestation.

One of the symptoms of this disease is the inability of the adult to fly, with the resulting appearance of many "crawlers" near the hive. This is believed to be due to the mechanical stoppage of gas exchange in these heavily infested tracheae which lead to the flight muscles. Infected bees may be observed falling from the alighting board and, in cool weather, gathered in small clusters near the hive. The bees usually die of exposure or starvation. Another symptom is the abnormal position of the wings of walking bees which appear "dislocated" as opposed to the normal hooked or flat positions.

For diagnosis, the sample should consist of 20 or more bees exhibiting the gross symptoms of "crawlers" or dislocated wings. Each bee is then pinned through its thorax, back side down, to a piece of cork or wax (fig. 16). Its head and front legs are then removed by pushing them off with a scalpel in a downward and forward motion which pulls forward the esophagus and exposes the great tracheal trunks in the mesothorax (fig. 17). These are then examined under a dissecting microscope. Healthy tracheae appear cream or white in color and contain air. With a heavy mite infestation, the tracheae contain brown blotches or they may be pitch black. If no discoloration or mites are noted, a continuing search should be made for the mite itself which may be found closer to the spiracular openings. In some cases all stages in the life cycle of the mite may be present in one honey bee.

A more accurate diagnosis can be made by removing the head and legs of the suspected bees and cutting the thorax in two pieces. The cut should be made crosswise with a sharp pair of scissors. Place the pieces of the thorax, containing the trachea, on the microscope slide and add a few drops of lactic acid. The acid makes the material more transparent

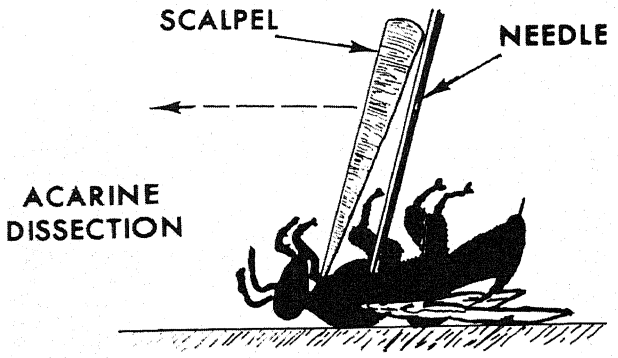


Figure 16.—Positioning the bee for dissection and diagnosis of acarine disease.

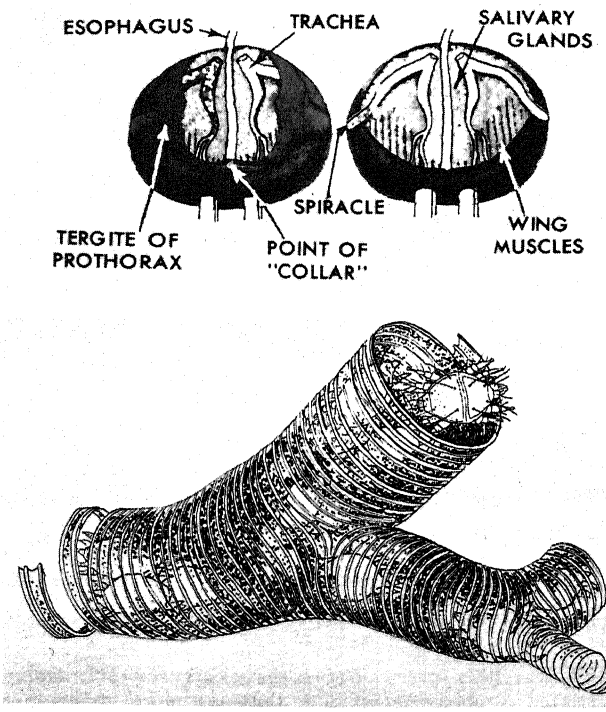


Figure 17.—Above, the location of the trachea in the thorax; below, sketch of the trachea containing acarine mites.

and also helps in removing the muscle. With the aid of a dissecting microscope carefully remove the muscles and draw out the tracheae. Mites are usually found in the end of the trachea closest to the exterior of the bees.

Fresh samples of bees yield the most desirable results. Bees should be examined within 2 or 3 days after being killed with ethyl ether or chloroform. If samples have to be held longer, use liquid of Oudemann (see composition in appendix) to store the bees.

Examination for External Mites—There is no damage caused by the external mites presently found in the United States. The following method has been used successfully in Laramie, Wyoming, and in Beltsville, Maryland.

1. Place 10-15 adult bees in a 50-ml vial and wet them with a 1:10,000 solution of Triton-X 100 or similar wetting agent.

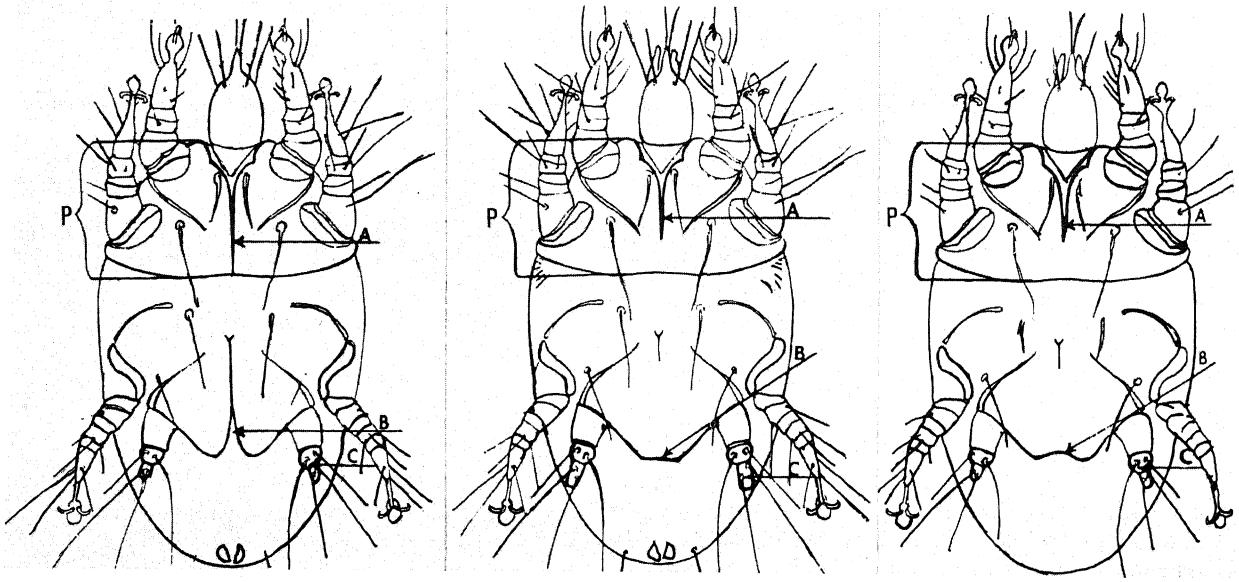
2. Add sufficient water to fill vial to about two-thirds of its capacity.

3. Shake vigorously and allow the tube to stand for 5-10 minutes.

4. Remove 1-2 ml of the liquid and place it on a watch glass or petri dish bottom for examination.

5. A dissecting microscope is sufficient to determine the presence or absence of the mites. However, if you desire to key the mites to species, you will need a microscope with an oil immersion objective (see fig. 18).

Asiatic Mites—One of the differences between *Acarapis woodi* and the Asiatic mites is their size. An oil immersion objective is required for seeing *A. woodi*, but both Asiatic mites, *Varroa jacobsoni* and *Tropilaelaps clareae*, can be seen without a microscope. Another difference is that the Asiatic mites can be found in sealed cells containing developing brood while *A. woodi* is found only in adult bees. The other major difference is one mentioned pre-



A. dorsalis

Apodeme (A) full length of propodosoma (P)

Coxal plate (B) deep indentation

Tarsal joints (C) <10 μm

A. externus

Apodeme (A) two-thirds length of propodosoma (P)

Coxal plate (B) truncate

Tarsal joints (C) > 10 μm

A. woodi

Apodeme (A) two-thirds length of propodosoma (P)

Coxal plate (B) shallow indentation

Tarsal joints (C) <10 μm

Figure 18.—Other species of mites that closely resemble *Acarapis woodi*, the internal tracheal mite, are found on honey bees. These are the so-called external mites *A. dorsalis* and *A. externus* which exhibit distinctive morphological characters that may be used in separating the three species. An oil immersion objective is necessary to see *A. woodi*, especially when making species differentiations.

viously: *A. woodi* is found internally while the Asiatic mites are external (fig. 19).

Varroa jacobsoni—This mite is found on young drones or workers, especially on the bee's thorax near the point where its wings are attached to its body. The mites can also be seen at the point of attachment of the thorax to the head or the thorax to the abdomen. In addition, *V. jacobsoni* infests brood cells and sealed or unsealed brood. Bees infested with *V. jacobsoni* may emerge wingless or with deformed wings.

Tropilaelaps clareae—This is the only parasitic mite of the honey bee that may have an alternate host, field rats. The mite feeds on living or dead larvae, pupae, and adult bees. The worker bees apparently seal the mites in the broad cells with the developing honey bees. Adults that survive the mite infestation may emerge with damaged wings.

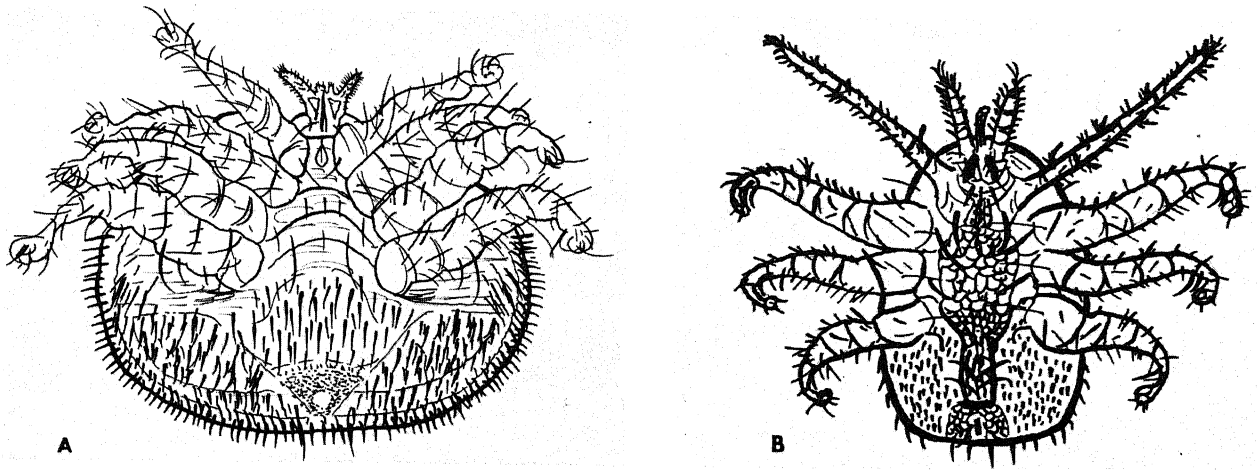


Figure 19.—Asiatic mites: (A) *Varroa jacobsoni*, (B) *Tropilaelaps clareae*.

WAX MOTH

There are two species of wax moths that neither cause a disease nor are parasitic on the individual bee, but are responsible for tremendous destruction to the colony. They are the greater wax moth, *Galleria mellonella*, and the lesser wax moth, *Achroia grisella* (fig. 20).

Colonies weakened by diseases or other causes are particularly susceptible to invasion and takeover by the larvae which eat the combs, honey, and pollen. The greater wax moth is most destructive to combs in storage, especially to combs in dark, warm, poorly ventilated places. The larvae of the moths are the destructive stage in both the greater and lesser wax moth. Damage by the greater wax moth is shown in figure 21. More information on the biology of the wax moth and its control is available in Farmers' Bulletin No. 2217.[2]

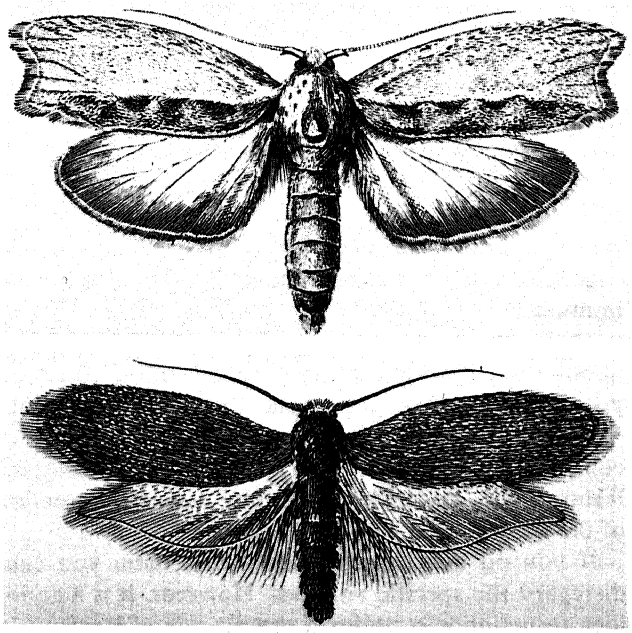


Figure 20.—The greater wax moth, *Galleria mellonella*, above, and the lesser wax moth, *Achroia grisella*, below. These species may easily be distinguished from each other by the shape of the wings and their comparative sizes. The greater wax moth is approximately two times larger than the lesser.

[2]U.S. Department of Agriculture. Controlling the greater wax moth; a pest of honeycombs. Farmers' Bulletin 2217. Rev. Oct. 1972. 10 pp. Washington, D.C.

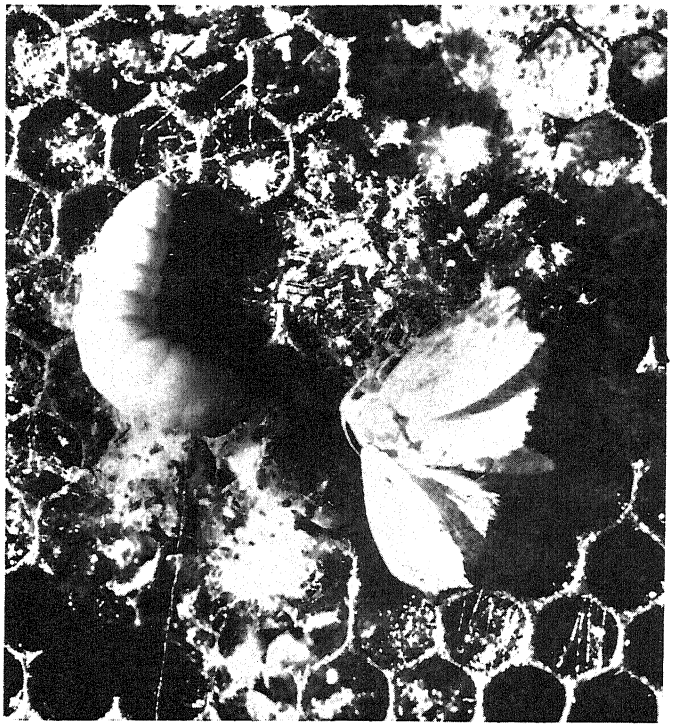


Figure 21.—Damage by the greater wax moth.

EXAMINATION OF HONEY FOR *BACILLUS LARVAE* SPORES

Occasionally it may be necessary to examine honey for the presence of *B. larvae*. Due to the high carbohydrate and other natural bacteriostatic substances in honey, the examination of honey requires special considerations. The following technique developed by A. P. Sturtevant is reported to recover about 84-89 percent of the spores.

Take 5 ml of honey and dilute it with 45 ml of distilled water in a centrifuge tube. Be sure that the honey is fully suspended before proceeding. Centrifuge the contents of the tube at 2,000 r/min for 45 minutes.

Discard the supernatant and leave a pellet of about 1 ml. If your centrifuge tube is not graduated, you should mark your tube for 0.1 and 1.0 ml. Resuspend the pellet in 45 ml of distilled water and centrifuge again at 2,000 r/min for 30 minutes. Remove the supernatant carefully and leave a pellet of 0.1 ml.

If you do not require quantitative data, you can disregard the specific volumes. However, it is a good idea to maintain a uniform sample size of honey.

Spores of *B. larvae* can be enumerated by a number of means, including methods used for counting *N. apis* spores. The method Sturtevant used was the Breed and Brew counting technique. This is basically a method in which a known volume of suspension is smeared on a premeasured area on the microscope slide. Dairy microbiologists used this technique for counting bacteria in milk. Breed slides may be available in some dairy laboratories or they can be made by etching a 1-cm^2 section on a standard microscope slide.

Using a standard loop or a pipet, place 0.01 ml of the suspension in the 1-cm^2 section. Spread the suspension uniformly while the smear is drying. Heat fix the smear before staining with a bacterial stain. If you have a microscope with an oil immersion phase objective, no staining is required. For ordinary light microscopes, a stained smear is a necessity. Be sure to determine the area of the microscopic field of the microscope objective that you will use for the counts. This can be calculated by measuring the radius of the microscopic field using a stage micrometer. The area of the field = πr^2 . The formula for counting the number of spores is as follows:

$$\text{No. spores/ml honey} = \frac{\text{Total No. spores counted} \times 100 \times D}{\text{No. fields counted} \times 5}$$

where

D = dilution factor.

In making direct microscopic counts of any bacteria, be especially careful of the shape of the organism. Not everything present on the smear is necessarily *B. larvae*. There is a tendency for *B. larvae* spores to clump, so count as many fields as you can.

There are no reliable methods for making plate counts of *B. larvae*. If necessary, biochemical confirmation of *B. larvae* can be made by conducting some simple tests which are routine to most bacteriology laboratories. (See table 2 for differentiation of *Bacillus* spp. found in honey bees.)

APPENDIX

The Microscope

Handling and Care

Always carry the microscope by the correct method. Grasp the arm of the scope with one hand and place the other hand under the base (see fig. 22).

Do **not** touch the glass parts of the microscope with your bare hands for this leaves a smudge which results in a blurred image of the specimen. To remove smudges from the microscope lens and prepared slides, wipe them with lens paper provided for this purpose. Do **not** use other cleaning materials on the optical parts.

Some Useful Terms

1. Depth of Focus —This is defined as the thickness of the specimen which may be seen in focus at one time. The greater the magnification, the thinner the layer in focus at one time; and conversely, the lesser the magnification, the thicker the layer in focus at one time.

2. Field of View —The visible area seen through the microscope when a specimen is in focus. This area varies with the objective; the greater the magnification, the smaller the field of view. The field of view is usually expressed in millimeter (mm) diameter.

Parts of the Microscope

1. **Eyepiece**—The uppermost optical lens system, sometimes referred to as the ocular. Look into the eyepiece and you may see a hairline pointer; also notice that when you turn the eyepiece the pointer moves. This pointer is an important part of the student microscope because it allows you to locate precisely an area or object about which you may wish to communicate with the instructor. This assures that both student and instructor are speaking about the same area or object. The eyepiece of your microscope magnifies ( ) times.

2. **Revolving Nosepiece**—The instructor will point out this part to which are attached three or four cylindrical parts called **Objectives.** The nosepiece will rotate 360° in either direction. When it is rotated a clicking sound is heard as each **Objective** is properly aligned.

3. **Objective**—The four objectives are the low power objective, which magnifies ( ) times; the medium power objective, which magnifies ( ) times; the high dry objective, which magnifies ( ) times; and the oil immersion objective, which magnifies ( ) times. To obtain the total magnification while using an objective, multiply the magnification of the eyepiece by the objective being used.

4. **Stage**—Beneath the objectives is a black plate, the **Stage,** to which the **Slide Clips** are attached. These clips are used to hold the glass microscope slide in place. A hole in the center of the stage allows light to pass from the **Illuminator** below up through the specimen being viewed.

5. **Condenser**—Just under the stage and attached to it is the **Condenser** with an **Iris Diaphragm.** This diaphragm has an arm which, when moved, changes the size of the opening, thereby regulating the

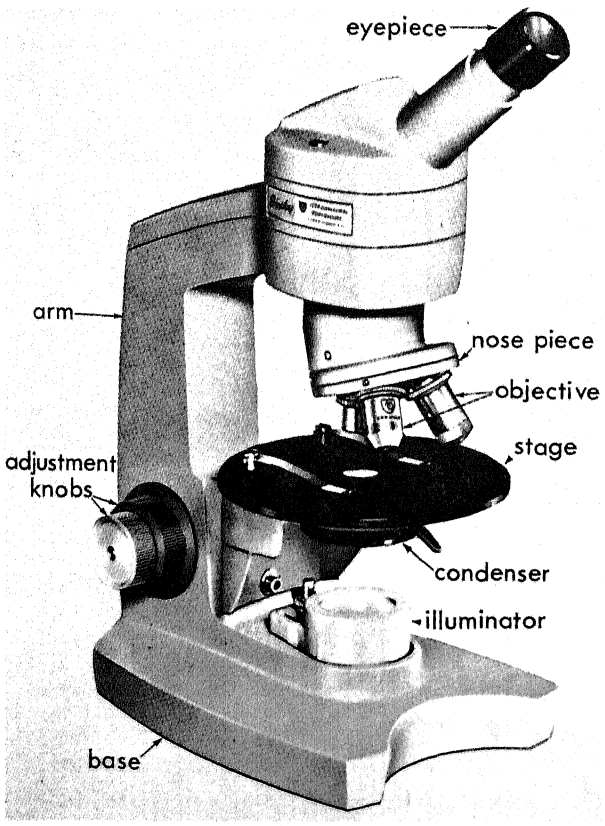


Figure 22.—Parts of the microscope.

amount of light passing through. A very dark or thick specimen will require more light than one that is thin or colorless.

6. **Adjustment Knob**—Between the **Carrying Arm** and the **Base** are two **Adjustment Knobs**, one for coarse and one for fine adjustments. The outer is for fine adjustment.

7. **Illuminator**—Built-in illuminators or microscope lamps with a mirror are necessary for examination of the specimen. Most microscopes are made for bright-field illumination. If you desire to examine a specimen by dark-field illumination, a special dark-field stop or star diaphragm must be inserted into the condenser.

Use of the Microscope

If you wear eyeglasses corrected only for near- or far-sightedness, you need not wear them while using a microscope. However, if your glasses have a correction for astigmatism it is desirable that they be worn, because eyestrain may result if they are not.

Locating the Subject

Center the slide on the stage of your microscope and under the stage clips. First use the low power objective with the coarse adjustment knob, then the fine adjustment knob, to finally bring the specimen into focus. You may want to increase the amount of light by opening the iris diaphragm wider. Center a portion of the specimen so it occupies the middle of the field before proceeding.

Next switch to the high dry objective ( ). The section of the specimen previously selected should still be in the center of the field and the microscope should require only a slight turn of the fine adjustment knob to bring it back into focus. If you cannot obtain a sharp image with only the fine adjustment, switch back to the low objective and repeat the procedure. **Do not use the coarse adjustment knob** with the high dry or the oil immersion objectives.

It is helpful, when referring to different objects in the field of view, to use the clock system for reference.

This method of locating a specimen or part of the specimen on a slide is the quickest and surest and should always be used. Never attempt to locate the specimen with the higher magnifying objectives; it takes too much time and could damage your microscope.

Use of the Oil Immersion Objective

Care must be exercised when using the objective so as not to damage the specimen slide or the objective. Proceed in the following manner. Focus onto the specimen progressively with the low power, medium power, and high dry objectives. Then fully raise the nosepiece by means of the coarse adjustment knob. Place a small drop of Crown's or Cargille's immersion oil (mineral oil or other substitutes should not be used) in the center of the circle of light formed on the specimen slide. Lower the oil immersion objective into the oil droplet and with the coarse, then the fine, adjustment knob, bring the specimen into sharp focus. Remember with this fine adjustment knob, the field of view is greatly reduced and less light is being transmitted; therefore you should open the iris diaphragm further.

Laboratory Cultivation of Micro-organisms Associated With Honey Bees

It is not possible to cite all the culture media that are available to culture micro-organisms found in diseased bees. The few shown here are those used by our laboratory.

Bacillus larvae: Brain heart infusion agar fortified with 0.1 g thiamin hydrochloride/liter.

Bacillus alvei, B. pulvifaciens, and *Pseudomonas apiseptica*: Nutrient agar.

Ascosphaera apis and other fungi: Sabouraud dextrose agar fortified with 2 g/liter of yeast extract.

Streptococcus pluton: Recovery of *S. pluton* from diseased larvae is facilitated by enrichment in a broth medium anaerobically for 24 to 36 hours at 34° C. Subsequent plating on freshly prepared media requires 36 to 48 hours incubation under the same conditions.

For *S. pluton* medium (after L. Bailey 1959)[3]: 10 g yeast extract (Difco), 10 g glucose, 100 ml 1M KH_2PO_4 (pH 6.6 adjusted with KOH), 20 g agar, distilled water to make 1000 ml. Autoclave at 10 lb/in^2 (116°C) for 20 min.

For *S. pluton* broth medium: As above, but with only 0.75 g agar per liter.

Alternatively, both media may be autoclaved at 15 lb/in^2 (121°C) for 15 min with no deleterious effects, provided the glucose is sterilized as a separate solution and returned during cooling.

Anaerobic jars: The "Gas Pak" (BBL) Anaerobic System employing a disposable hydrogen and carbon dioxide generator is used. Fresh palladium catalyst is suggested with each use. Renewal of the catalyst is accomplished by heating to 160-170°C for 2 or more hours. Anaerobic conditions are indicated by reduction of a methylene blue saturated indicator strip (disposable anaerobic indicator, BBL).

[3]Bailey, L. An improved method for the isolation of *Streptococcus pluton* and observations on its distribution and ecology. J. Insect Path. 1:80-85. 1959.